In one embodiment, the immune effector cells are T cells. In a separate embodiment, the immune effector cells can be genetically modified by transduction with a transgene coding for example, IL-2, IL-11 or IL-13. Methods for introducing transgenes *in vitro*, *ex vivo* and *in vivo* are well known in the art. See Sambrook et al. (1989) *supra*.

An effector cell population suitable for use in the methods of the present invention can be autologous or allogeneic, preferably autologous. When effector cells are allogeneic, preferably the cells are depleted of alloreactive cells before use. This can be accomplished by any known means, including, for example, by mixing the allogeneic effector cells and a recipient cell population and incubating them for a suitable time, then depleting $CD69^+$ cells, or inactivating alloreactive cells, or inducing anergy in the alloreactive cell population.

Hybrid immune effector cells can also be used. Immune effector cell hybrids are known in the art and have been described in various publications. See, for example, International Patent Application Nos. WO 98/46785 and WO 95/16775.

The effector cell population can comprise unseparated cells, i.e., a mixed population, for example, a PBMC population, whole blood, and the like. The effector cell population can be manipulated by positive selection based on expression of cell surface markers, negative selection based on expression of cell surface markers, stimulation with one or more antigens *in vitro* or *in vivo*, treatment with one or more biological modifiers *in vitro* or *in vivo*, subtractive stimulation with one or more antigens or biological modifiers, or a combination of any or all of these.

Effector cells can obtained from a variety of sources, including but not limited to, PBMC, whole blood or fractions thereof containing mixed populations, spleen cells, bone marrow cells, tumor infiltrating lymphocytes, cells obtained by leukopharesis, biopsy tissue, lymph nodes, e.g., lymph nodes draining from a tumor. Suitable donors include an immunized donor, a non-immunized (naïve) donor, treated or untreated donors. A "treated" donor is one that has been

exposed to one or more biological modifiers. An "untreated" donor has not been exposed to one or more biological modifiers.

Methods of extracting and culturing effector cells are well known. For example, effector cells can be obtained by leukopharesis, mechanical apheresis using a continuous flow cell separator. For example, lymphocytes and monocytes can be isolated from the buffy coat by any known method, including, but not limited to, separation over Ficoll-Hypaque™ gradient, separation over a Percoll gradient, or elutriation. The concentration of Ficoll-Hypaque™ can be adjusted to obtain the desired population, for example, a population enriched in T cells. Other methods based on affinity are known and can be used. These include, for example, fluorescence-activated cell sorting (FACS), cell adhesion, magnetic bead separation, and the like. Affinity-based methods may utilize antibodies, or portions thereof, which are specific for cell-surface markers and which are available from a variety of commercial sources, including, the American Type Culture Collection (Manassas, VA). Affinity-based methods can alternatively utilize ligands or ligand analogs, of cell surface receptors.

The effector cell population can be subjected to one or more separation protocols based on the expression of cell surface markers. For example, the cells can be subjected to positive selection on the basis of expression of one or more cell surface polypeptides, including, but not limited to, "cluster of differentiation" cell surface markers such as CD2, CD3, CD4, CD8, TCR, CD45, CD45RO, CD45RA, CD11b, CD26, CD27, CD28, CD29, CD30, CD31, CD40L; other markers associated with lymphocyte activation, such as the lymphocyte activation gene 3 product (LAG3), signaling lymphocyte activation molecule (SLAM), T1/ST2; chemokine receptors such as CCR3, CCR4, CXCR3, CCR5; homing receptors such as CD62L, CD44, CLA, CD146, $\alpha 4\beta 7$, $\alpha E\beta 7$; activation markers such as CD25, CD69 and OX40; and lipoglycans presented by CD1. The effector cell population can be subjected to negative selection for depletion of non-T cells and/or particular T cell subsets. Negative selection can be performed on the basis of cell surface expression of a variety of molecules, including, but not limited to,

B cell markers such as CD19, and CD20; monocyte marker CD14; the NK cell marker CD56.

An effector cell population can be manipulated by exposure, *in vivo* or *in vitro*, to one or more biological modifiers. Suitable biological modifiers include, but are not limited to, cytokines such as IL-2, IL-4, IL-10, TNF-α, IL-12, IFN-γ; non-specific modifiers such as phytohemagglutinin (PHA), phorbol esters such as phorbol myristate acetate (PMA), concanavalin-A, and ionomycin; antibodies specific for cell surface markers, such as anti-CD2, anti-CD3, anti-IL2 receptor, anti-CD28; chemokines, including, for example, lymphotactin. The biological modifiers can be native factors obtained from natural sources, factors produced by recombinant DNA technology, chemically synthesized polypeptides or other molecules, or any derivative having the functional activity of the native factor. If more than one biological modifier is used, the exposure can be simultaneous or sequential.

The present invention provides compositions comprising immune effector cells, which may be T cells, enriched in antigen-specific cells, specific for a peptide of the invention. By "enriched" is meant that a cell population is at least about 50-fold, more preferably at least about 500-fold, and even more preferably at least about 5000-fold or more, enriched from an original naive cell population. The proportion of the enriched cell population which comprises antigen-specific cells can vary substantially, from less than 10% up to 100% antigen-specific cells. If the cell population comprises at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90%, antigen-specific immune effector cells, specific for a peptide of the invention, then the population is said to be "substantially pure". The percentage which are antigen-specific can readily be determined, for example, by a ^{3}H-thymidine uptake assay or cytokine release assay in which the effector cell population (for example, a T-cell population) is challenged by an antigen-presenting matrix presenting an antigenic peptide of the invention.

Assaying Antigen-Specificity

An *in vitro* system will be needed to test or confirm which version of the modified or heterologous tumor antigen is most likely to be immunogenic in humans or the test subject. In this system, DCs will be used to present antigen to autologous peripheral blood lymphocytes. The DCs can be pulsed or transduced. Various culture conditions have been described that will support the generation of effector cells in cultures of DCs and lymphocytes. After several rounds of stimulation, the effector cells generated are tested for their ability to recognize native tumor antigen. Fewer rounds of stimulation may be required for antigens with high immunogenic potential. Both T helper ($CD4^+$) and cytolytic effector cells ($CD8^+$) can be elicited. The development of TAA-specific cells can be measured by several methods including proliferation or cytokine production (e.g. TNF-α, interferon-γ) upon exposure to TAA or lysis of TAA-expressing target cells as assessed by release of various intracellular labels/markers such as ^{51}Chromium or lactose dehydrogenase (LDH). The antigen that induces the strongest response (in particular cytolytic activity) against the native human antigen would then be selected for immunization purposes.

In addition to previously identified and characterized antigens and epitopes, the methods of this invention also can use newly identified antigens which can be identified as exemplified below.

Production of Epitope or Antigen

Most preferably, heterologous/altered antigens and peptides of the present invention can be synthesized using an appropriate solid state synthetic procedure. Steward and Young, Solid Phase Peptide Synthesis, Freemantle, San Francisco, Calif. (1968). A preferred method is the Merrifield process. Merrifield, Recent Progress in Hormone Res. **23**:451 (1967). The antigenic activity of these peptides may conveniently be tested using, for example, the assays as described herein.

Once an isolated peptide of the invention is obtained, it may be purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any

other standard technique for protein purification. For immunoaffinity chromatography, an epitope may be isolated by binding it to an affinity column comprising antibodies that were raised against that peptide, or a related peptide of the invention, and were affixed to a stationary support.

Alternatively, affinity tags such as hexa-His (Invitrogen), Maltose binding domain (New England Biolabs), influenza coat sequence (Kolodziej et al. (1991) Methods Enzymol. **194**:508), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. Isolated peptides can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance, and x-ray crystallography.

Also included within the scope of the invention are antigenic peptides that are differentially modified during or after translation, e.g., by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand, (Ferguson et al. (1988) Ann. Rev. Biochem. **57**:285).

Another aspect of the invention encompasses isolated nucleic acid sequences that encode the novel antigenic peptides described herein. With regard to nucleic acid sequences of the present invention, "isolated" means: an RNA or DNA polymer, portion of genomic nucleic acid, cDNA, or synthetic nucleic acid which, by virtue of its origin or manipulation: (i) is not associated with all of a nucleic acid with which it is associated in nature (e.g. is present in a host cell as a portion of an expression vector); or (ii) is linked to a nucleic acid or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature. By "isolated" it is further meant a nucleic acid sequence: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) synthesized by, for example, chemical synthesis; (iii) recombinantly produced by cloning; or (iv) purified, as by cleavage and gel separation.

The nucleic acid sequences of the present invention may be characterized, isolated, synthesized and purified using no more than ordinary skill. See Sambrook et al. (1989) *supra*.

Compositions

This invention also provides compositions containing any of the above-mentioned proteins, muteins, polypeptides, nucleic acid molecules, vectors, cells antibodies and fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a "pharmaceutically acceptable carrier" for diagnostic and therapeutic use. These compositions also can be used for the preparation of medicaments for the diagnosis and treatment of diseases such as cancer.

Tumor Protection in Animal Models

The murine B16 melanoma model was used. In this model, C57BL/6 mice were immunized with bone marrow-derived DCs transduced with an Ad vector encoding either human gp100 (Ad/hugp100) or mouse gp100 (Ad/mgpl00). Mice immunized against heterologous human gp100 developed a protective immune response and were resistant to a lethal subcutaneous challenge of B16 melanoma cells (syngeneic tumor cell line that expresses gp100). In contrast, mice immunized with homologous mouse gp100 failed to mount a protective immune response against B16 melanoma cells and developed tumors at the site of B16 cell injection. This finding illustrates the difficulty in breaking tolerance against a self antigen (mouse gpl00). The corresponding heterologous antigen from a different species (human gp100), however, is likely to contain several Class I and Class II-associated epitopes that will be recognized as foreign and elicit $CD8^+$ and $CD4^+$ T cell responses, respectively. The induction of cross-reactive CTLs that recognize both the heterologous and homologous self-antigen can then lead to lysis of host tumor cells.

Unfortunately, this type of animal model cannot be used to test the efficacy of modified or heterologous tumor antigens being considered for use in humans since mice and humans recognize different epitopes, primarily as a result of differences in their MHC molecules. It may be possible, however, to use the allogeneic human peripheral blood lymphocyte - severe combined immunodeficiency mouse (Hu-PBL-SCID) model. SCID mice lack mature B and

T lymphocytes and can be reconstituted with human PBLs. It may be possible to immunize such mice with test antigen to induce a response in adoptively transferred human PBLs and evaluate protection against challenge with a human tumor cell line (Mosier et al. (1988) Nature **335**:256; Parney et al. (1997) Human Gene Therapy **8**:1073; and Albert et al. (1997) J. Immunol. **159**:1393).

Another possibility is immunization of HLA-A2.1 transgenic mice to reproduce the immune reactivity of HLA-A2 individuals (Wentworth et al. (1996) Eur. J. Immunol. **26**:97).

Vectors Useful in Genetic Modifications

In general, genetic modifications of cells employed in the present invention are accomplished by introducing a vector containing a polypeptide or transgene encoding a heterologous or an altered antigen. A variety of different gene transfer vectors, including viral as well as non-viral systems can be used. Viral vectors useful in the genetic modifications of this invention include, but are not limited to adenovirus, adeno-associated virus vectors, retroviral vectors and adeno-retroviral chimeric vectors. APC and immune effector cells can be modified using the methods described below or by any other appropriate method known in the art.

Construction of Recombinant Adenoviral Vectors or Adeno-Associated Virus Vectors

Adenovirus and adeno-associated virus vectors useful in the genetic modifications of this invention may be produced according to methods already taught in the art. (see, *e.g.*, Karlsson et al. (1986) EMBO 5:2377; Carter (1992) Current Opinion in Biotechnology **3**:533; Muzcyzka (1992) Current Top. Microbiol. Immunol. **158**:97; and GENE TARGETING: A PRACTICAL APPROACH (1992) ed. A. L. Joyner, Oxford University Press, NY). Several different approaches are feasible. Preferred is the helper-independent replication deficient human adenovirus system.

The recombinant adenoviral vectors based on the human adenovirus 5 (Virology **163**:614, 1988) are missing essential early genes from the adenoviral genome (usually E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products *in trans*. In place of the missing adenoviral genomic sequences, a transgene of interest can be cloned and expressed in cells infected with the replication deficient adenovirus. Although adenovirus-based gene transfer does not result in integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), and therefore is not stable, adenoviral vectors can be propagated in high titer and transfect non-replicating cells. Human 293 cells, which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines. However, other cell lines which allow replication-deficient adenoviral vectors to propagate therein can be used, including HeLa cells.

Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M.S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) VIROLOGY, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham F. et al., pp. 109-128 in METHODS IN MOLECULAR BIOLOGY, Vol. 7: GENE TRANSFER AND EXPRESSION PROTOCOLS, Murray E. (ed.) Humana Press, Clifton, N.J. (1991); Miller N. et al. (1995) FASEB Journal **9**:190; Schreier H (1994) Pharmaceutica Acta Helvetiae **68**:145; Schneider and French (1993) Circulation **88**:1937; Curiel D.T. et al. (1992) Human Gene Therapy **3**:147; Graham F.L. et al., WO 95/00655; Falck-Pedersen WO 95/16772; Denefle P. et al. WO 95/23867; Haddada H. et al. WO 94/26914; Perricaudet M. et al. WO 95/02697; and Zhang W. et al. WO 95/25071. A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996). See also, the papers by Vile et al. (1997) Nature Biotechnology **15**:840; and Feng et al. (1997) Nature Biotechnology. **15**:866, describing the construction and use of adeno-retroviral chimeric vectors that can be employed for genetic modifications.

Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Kotin R. (1994) Human Gene Therapy **5**:793; Flotte T.R. et al. (1995) Gene Therapy **2**:357; Allen J.M. WO 96/17947; and Du et al. (1996) Gene Therapy **3**:254.

APCs can be transduced with viral vectors encoding a relevant antigen. The most common viral vectors include recombinant poxviruses such as vaccinia and fowlpox virus (Bronte et al. (1997) PNAS **94**:3183; and Kim et al. (1997) J. Immunother. **20**:276) and, preferentially, adenovirus (Arthur et al. (1997) J. Immunol. **159**:1393; Wan et al. (1997) Human Gene Therapy **8**:1355; and Huang et al. (1995) J. Virol. **69**:2257). Retrovirus also may be used for transduction of human APCs (Marin et al. (1996) J. Virol. **70**:2957).

In vitro/ex vivo, exposure of human DCs to adenovirus (Ad) vector at a multiplicity of infection (MOI) of 500 for 16-24 hours in a minimal volume of serum-free medium reliably gives rise to transgene expression in 90-100% of DCs. The efficiency of transduction of DCs or other APCs can be assessed by immunofluorescence using fluorescent antibodies specific for the tumor antigen being expressed (Kim et al. (1997) *J. Immunother.* **20**:276). Alternatively, the antibodies can be conjugated to an enzyme (e.g. HRP) giving rise to a colored product upon reaction with the substrate. The actual amount of antigen being expressed by the APCs can be evaluated by ELISA.

Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

In vivo transduction of DCs, or other APCs, can be accomplished by administration of Ad (or other viral vectors) via different routes including intravenous, intramuscular, intranasal, intraperitoneal or cutaneous delivery. The preferred method is cutaneous delivery of Ad vector at multiple sites using a total dose of approximately $1x10^{10}$-$1x\ 10^{12}$ i.u. Levels of *in vivo* transduction can be roughly assessed by co-staining with antibodies directed against APC marker(s) and the antigen being expressed. The staining procedure can be carried out on biopsy samples from the site of administration or on cells from draining lymph

nodes or other organs where APCs (in particular DCs) may have migrated (Condon et al. (1996) Nature Med. **2**:1122; Wan et al. (1997) Human Gene Therapy **8**:1355). The amount of antigen being expressed at the site of injection or in other organs where transduced APCs may have migrated can be evaluated by ELISA on tissue homogenates.

Although viral gene delivery is more efficient, DCs can also be transduced *in vitro*/*ex vivo* by non-viral gene delivery methods such as electroporation, calcium phosphate precipitation or cationic lipid/plasmid DNA complexes (Arthur et al. (1997) Cancer Gene Therapy **4**:17). Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

In vivo transduction of DCs, or other APCs, can potentially be accomplished by administration of cationic lipid/plasmid DNA complexes delivered via the intravenous, intramuscular, intranasal, intraperitoneal or cutaneous route of administration. Gene gun delivery or injection of naked plasmid DNA into the skin also leads to transduction of DCs (Condon et al. (1996) Nature Med. **2**:1122; and Raz et al. (1994) PNAS **91**:9519). Intramuscular delivery of plasmid DNA may also be used for immunization (Rosato et al. (1997) Human Gene Therapy **8**:1451.

The transduction efficiency and levels of transgene expression can be assessed as described herein.

Administration Methods

Dendritic cells derived from peripheral blood of a subject such as a human patient are transduced with adenovirus vector encoding the tumor antigen using a multiplicity of infection of 200-500. Approximately 24 hours after infection, the transfected dendritic cells (10×10^7 cells) are administered to the patient iv or subcutaneously. The process is repeated 3-4 weeks later with up to 6 administrations of dendritic cells. Since it is possible to freeze dendritic cells and administer thawed cells, the subject does not have to be leukopharesed each time.

The agents identified herein as effective for their intended purpose can be administered to subjects having tumors or to individuals susceptible to or at risk of developing a tumor by inducing an immune response against the tumor. When the agent is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject. To determine patients that can be beneficially treated, a tumor regression can be assayed. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the therapy.

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

More particularly, an agent of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including nasal, topical (including transdermal, aerosol, buccal and sublingual), parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

Adoptive Immunotherapy and Vaccines

The expanded populations of antigen-specific immune effector cells of the present invention also find use in adoptive immunotherapy regimes and as

vaccines. Thus, tumors expressing the antigen can be eradicated using the methods and compositions described herein.

Adoptive immunotherapy methods involve, in one aspect, administering to a subject a substantially pure population of educated, antigen-specific immune effector cells made by culturing naïve immune effector cells with APCs as described above. Preferably, the APCs are dendritic cells. In one embodiment, the adoptive immunotherapy methods described herein are autologous. In this case, the APCs are made using cells isolated from a single subject. The expanded population also employs T cells isolated from that subject. Finally, the expanded population of antigen-specific cells is administered to the same patient.

In a further embodiment, APCs or immune effector cells are administered with an effective amount of a stimulatory cytokine, such as IL-2 or a co-stimulatory molecule.

The following examples are intended to illustrate, but not limit the invention.

Experimental Examples

Animals and cell lines

Female C57BL/6 mice were purchased from Taconic (Germantown, NY) and were used at 8-12 weeks of age. Syngeneic SV40-transformed fibroblasts (SVB6KHA) have been described elsewhere (Gooding L.R. (1979) J. Immunol. **122:**1002) and were a gift from Dr. Linda Gooding (Emory University, Atlanta, GA). The B16.F10 melanoma cell line syngeneic to C57BL/6 mice was obtained from the National Cancer Institute (Bethesda, MD). For injection, B16.F10 cells ($1.5\text{-}2\times10^4$ cells) were resuspended in phosphate-buffered saline (PBS) and delivered to the abdomen subcutaneously (s.c.) in a 100 µl volume. Tumor size was measured with electronic digital calipers 3 times per week starting around day 10. Tumors ≥ 3 mm^2 in size were scored as positive.

Adenoviral vectors

All recombinant adenovirus (Ad) vectors used were derived from Ad serotype 2 from which the E1 region was deleted and replaced with an expression cassette containing a cytomegalovirus (CMV) promoter driving expression of the transgene. The vector encoding β-Galactosidase (Ad2/βGal-4) and human gp100 (Ad2/hugp100vl) contained intact E3 and E4 regions (Armentano D. (1997) J. Virol. **71**:2408 and Zhai Y. (1996) J. Immunol. **156**:7001. The vector encoding murine gp100 (Ad2/mgp100) or vector lacking a transgene (Ad2/empty vector), possessed an intact E3 region with an E4 region modified by removal of all open reading frames and replacement with the E4 open reading frame 6 and protein IX moved from its original location (Armentano D. (1985) Human Gene Therapy **6**:1343). Finally, the Ad vector encoding murine tyrosinase-related protein 2 (Ad2/mTRP-2) contained an intact E4 region but was deleted for E3. The E2 region was left intact in all vectors.

Adenoviral particles were gradient-purified as previously described (Armentano D. (1985) Human Gene Therapy **6**:1343) and titers were determined by end-point dilution on 293 cells using fluorescent isothiocyanate (FITC)-conjugated anti-hexon antibody (Rich D.P. (1993) Human Gene Therapy **4**:461).

Preparation of bone marrow-derived dendritic cells

Dendritic cells (DCs) were prepared from bone marrow essentially as described by Inaba et al. (Inaba K. (1992) J. Exp. Med. **176**:1693). Briefly, bone marrow was flushed from the tibias and femurs of C57BL/6 mice and depleted of erythrocyte with commercial lysis buffer (Sigma, St. Louis, MO). Bone marrow cells were then treated with a cocktail of antibodies (Pharmingen, San Diego, CA) directed against CD8 (clone 53-6.7), CD4 (clone GK1.5), CD45R/B220 (clone RA3-6B2), Ly-6G/Gr-1 (clone RB6-8C5) and Ia (clone KH74) followed by rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) to deplete lymphocytes, granulocytes and Ia^+ cells. The remaining cells were cultured for 6 days in 6-well plates in RPMI-1640 medium (Gibco, Grand Island,

NY) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% fetal calf serum (FCS) and 100 ng/ml recombinant mouse GM-CSF (Genzyme, Cambridge, MA). Loosely adherent DCs were then collected, replated in 100 mm dishes and cultured in the same medium for another 24 hours after removal of contaminating non-adherent cells. This final DC population was then collected for FACS analysis and transduction with Ad vector.

For analysis of surface markers, DCs were first incubated with unlabeled antibodies (Pharmingen) specific for the major histocompatibility complex (MHC) Class I (clone AF6-88.5) and Class II (clone AF6-120.1) molecules, the co-stimulatory molecules B7.1 (CD80; clone IG10) and B7.2 (CD86; clone GL-1), the adhesion molecule ICAM-1 (CD54; clone 3E2), the integrin CD 11 c (clone 3E2) and the myeloid surface marker CD13 (clone R3-242). The cells were then counterstained with FITC-conjugated antibodies specific for the primary antibody. FACS analysis of the stained cells was performed on an EPICS Profile Analyzer from Coulter.

Transduction of DCs with Ad vector was conducted in 6-well plates with $4x10^6$ DCs/well in a 3 ml volume of RPMI-1640 medium containing 10% FCS and 100 ng/ml GM-CSF. Virus was added to the wells at a multiplicity of infection (MOI) of 500 and the DCs were collected after 18-24 hours of incubation. For injection, transduced DCs were washed and resuspended in a 100 μl volume of PBS and delivered either s.c. to the abdomen or intravenously (i.v.) into the tail vein as specified in the text.

Cytotoxic T cell assay

To evaluate levels of cytotoxic T lymphocyte (CTL) activity, spleen cells from mice in the same treatment group (3 mice/group) were pooled and stimulated *in vitro* with syngeneic SVB6KHA fibroblasts transduced with Ad2 vector at an MOI of 100 for 24 hours. Cells were cultured in 24-well plates containing $5x10^6$ spleen cells and $0.8\text{-}1.5x10^5$ stimulator fibroblasts per well in a 2 ml volume. Cytolytic activity was assayed after 6 days of incubation. Target cells consisted of B16 melanoma cells and fibroblasts untransduced or transduced with virus at

an MOI of 100 for 48 hours. Targets were treated with 100 U/ml recombinant mouse γ-interferon (Genzyme) for 24 hours labeled with ^{51}Chromium (51-Cr; New England Nuclear) overnight (30 μCi/10^5 cells) and plated in round bottom 96 well plates at $5x10^3$ cells/well. Effector cells were added at various effector:target (E:T) cell ratios in triplicate. The total reaction volume was kept constant at 200 μl/well. After 5 hours of incubation of effector and target cells at 37°C/5% CO_2, 25 μl of cell-free supernatant was collected from each well and counted in a MicroBeta Trilux Liquid Scintillation Counter (Wallac Inc., Gaithersburg, MD). The amount of ^{51}Cr spontaneously released was obtained by incubating target cells in medium alone and the total amount of ^{51}Cr incorporated was determined by adding 1% Triton X-100 in distilled water. The percentage lysis was calculated as follows:

$$\% \text{ Lysis} = \frac{(\text{Sample cpm}) - (\text{Spontaneous cpm})}{(\text{Total cpm}) - (\text{Spontaneous cpm})} \times 100$$

ELISPOT assay

The frequency of splenic T lymphocytes reactive with gp100 was evaluated in an ELISPOT assay. Spleen cells from mice immunized with Ad2/hugp100- or Ad2/empty vector-transduced DCs (4 mice/group) were pool and stimulated with H-2^b-restricted CTL epitopes derived from human gp100 (KVPRNQDWL), murine gp100 (EGSRNQDWL) or ovalbumin as a negative control (SIINFEKL). (For human and murine gp100 peptides, see Overwijk et al. (1998) J. Exp. Med. **188**: 277-286; for ovalbumin peptide, see Brossart et al. (1997) J. Immunol. **158**: 3270-3276.)

After 4 hours of stimulation with peptide, the spleen cells were transferred to 96-well nitrocellulose filter plates coated with γ-interferon-specific antibodies. After 40 hours of incubation, the cells were removed by washing and biotinylated antibodies against γ-interferon were added to the wells. The subsequent addition of streptavidin-alkaline phosphatase gave rise to dark spots corresponding to γ-interferon-producing cells.

Experimental Results

Characterization of bone-marrow derived dendritic cells and transduction by adenovirus vectors

Dendritic cells (DCs) derived from mouse bone marrow exhibited the veiled dendrite morphology typical of DCs and displayed a characteristic set of DC surface markers (Crowley M. (1989) Cell. Immunol. **118**:108) as determined by FACS analysis (Table 2). The cells expressed high levels of the major histocompatibility (MHC) Class I and Class II molecules, the co-stimulatory molecules B7.1 and B7.2, the ICAM-I adhesion molecule, the integrin CD11c and the CD13 myeloid surface marker. Transduction of DCs with recombinant Ad2-based vectors was achieved reproducibly with an efficiency of 90% or greater. Transduction did not affect the distribution of DC surface markers significantly except for a reproducible increase in levels of MHC Class I molecules (Table 2).

Table 2. FACS analysis of dendritic cell surface markers

DC sample	B7.1	B7.2	MHC I	MHC II	ICAM1	CD11c	CD13
Untransduced	80	77	41	70	96	81	80
Transduced	84	83	85	79	94	71	74

Results shown are the percentage of bone marrow-derived DCs staining positive for each marker.
DCs were untransduced or transduced with Ad2/βGal-4.

Induction of tumor-specific cytotoxic T lymphocyte response by transduced dendritic cells

The ability of DCs to induce a cytotoxic T lymphocyte (CTL) response against a melanoma-associated antigen (MAA) was evaluated *in vivo*. DCs were transduced with an Ad vector encoding human gp100 (Ad2/hugp100vl), a differentiation antigen that is expressed by most melanomas but is also present in normal melanocytes and pigmented cells of the retina. Ad2/hugp100vl-

transduced DCs ($5x10^5$) were administered intravenously (i.v.) to C57BL/6 mice and, 15 days later, spleens were collected for assessment of CTL activity. Separate groups of mice were also treated with vehicle as a negative control or with the Ad2/hugp100v1 vector itself for comparison. The vector was delivered under conditions previously determined to be optimal for immunization (3×10^9 i.u. intradermally).

After *in vitro* re-stimulation with syngeneic fibroblasts transduced with Ad2/hugp100v1, effector splenocytes were tested for cytolytic activity against ^{51}Cr-labeled target fibroblasts that were either untransduced or transduced with Ad2/hugp100v1 or wild-type (WT) E3-deleted Ad (Ad2Δ2.9). The CTLs were also tested against B16 tumor cells, a cell line originally derived from a spontaneously arising melanoma in C57BL/6 mice which expresses the murine equivalent of human gp100.

As expected, mice treated with vehicle failed to develop any significant CTL activity against any of the targets (Figure 2A). Mice immunized with transduced DCs developed high levels of CTL activity against target fibroblasts infected with the Ad2/hugp100v1 vector. Interestingly, the bulk of the CTL response appeared to be directed against the hugp100 transgene product rather than adenoviral protein(s) since there was very little lysis of fibroblasts infected with WT Ad (Figure 2B).

Mice immunized i.d. with the Ad2/hugp100v1 vector itself, developed robust but comparatively lower levels of CTL activity against Ad2/hugp100v1-transduced fibroblasts. Furthermore, in contrast to the response obtained with transduced DCs, a significant proportion of the CTL response appeared to be specific for Ad antigen as indicated by the greater level of lysis of fibroblasts infected with WT Ad (Figure 2C). Importantly, CTLs from mice immunized with transduced DCs and, to a lesser extent, with Ad vector, were both able to lyse B16 tumor cells indicating that the CTLs raised against human gp100 also recognized the endogenous mouse gp100 expressed by the tumor cells (Figures 2B and 2C).

Immunization with Ad Vector-Transduced DCs Induces Anti-Tumor Protection

Groups of 5 C57BL/6 mice were immunized against the gp100 melanoma antigen with an intravenous injection of 5 x 10^5 bone marrow-derived dendritic cells (DCs) transfected with adenovirus vector encoding mouse gp100 (Ad2/mgp 100 DCs) or human gp100 (Ad2/hugp100 DCs). Uninfected DCs served as a negative control. Two weeks after immunization, the mice were challenged with a subcutaneous injection of 2 x 10^4 B16 melanoma cells and tumor growth was monitored over time. The results which are shown in Figures 1A through 1D and 3 indicate that immunization of the mice with the heterologous human gp100 antigen was more effective than immunization with the homologous mouse gp100 antigen in inducing protective immunity against B16 melanoma cells.

Cross-reactivity between human and murine gp100 CTL epitopes

ELISPOT analysis on spleen cells from mice immunized with Ad2/hugp100-transduced DCs confirmed the presence of splenic T lymphocytes specific for a dominant CTL epitope from human gp100 (Figure 4). Importantly, the CTLs, which were raised against hugp100, showed cross-reactivity against the corresponding murine gp100 epitope. This finding is in agreement with the observation of cross-reactivity at the CTL level (Figure 2) and the resistance of human gp100-immunized mice to challenge with B16 melanoma cells positive for murine gp100 (Figures 1A through 1D and 3).

As expected, spleen cells from mice immunized with Ad2/hugp100-transduced DCs did not display any significant reactivity against a known CTL epitope from ovalbumin and spleen cells from mice that received DCs transduced with Ad2/empty vector did not show any significant reactivity against any of the peptides (Figure 4).

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the following examples are intended to illustrate and not limit the scope of the invention. For example, any of the above-noted compositions and/or methods can

be combined with known therapies or compositions. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

CLAIMS

1. A substantially pure population of educated, antigen-specific immune effector cells produced by culturing naïve immune effector cells with antigen-presenting cells (APCs) cells which express a heterologous or an altered antigen distinct from the corresponding native self-antigen.

2. The population of claim 1, wherein the antigen presenting cells (APCs) are dendritic cells.

3. The population of claim 1, wherein the immune effector cells are cytotoxic T lymphocytes (CTLs).

4. The population of claim 1, wherein the APC have been genetically modified.

5. The population of claim 1, wherein immune effector cells have been genetically modified.

6. The population of claim 4, wherein the antigen-presenting cells comprise an exogenously added polynucleotide encoding the heterologous or altered antigen.

7. A composition comprising the population of claim 1 and a carrier.

8. The composition of claim 7, wherein the carrier is a pharmaceutically acceptable carrier.

9. The method of claim 1, wherein the self-antigen is selected from the group consisting of gp100, MART1, MUC1, HER-2, CEA, PSA, prostate

membrane specific antigen (PSMA), tyrosinase, tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2), NY-ESO-1, and GA733.

10. A method of inducing an immune response to a native self-antigen in a subject, comprising administering to the subject an effective amount of a heterologous or altered antigen corresponding to the native self-antigen and under the conditions that induce an immune response to the native self-antigen.

11. The method of claim 10, further comprising administering an effective amount of a cytokine to the subject.

12. The method of claim 10, further comprising administering an effective amount of a co-stimulatory molecule to the subject.

13. The method of claim 10, wherein more than one heterologous or altered antigen that induces an immune response to the native or self-antigen.

14. The method of claim 10, wherein the native self-antigen is a tumor antigen.

15. The method of claim 14, wherein the tumor antigen is selected from the group consisting of gp100, MART1, MUC1, HER-2, CEA, PSA, prostate membrane specific antigen (PSMA), tyrosinase, tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2), NY-ESO-1, and GA733.

16. A method of inducing an immune response to a native self-antigen in a subject, comprising administering to the subject an effective amount of an antigen-presenting cell expressing a heterologous or altered antigen corresponding to the native self-antigen and under conditions that induce an immune response to the self-antigen in the subject.

17. The method of claim 16, further comprising administering an effective amount of a cytokine to the subject.

18. The method of claim 16, further comprising administering an effective amount of a co-stimulatory molecule to the subject.

19. The method of claim 16, wherein the antigen is a tumor antigen.

20. The method of claim 16, wherein the antigen-presenting cell is a dendritic cell.

21. The method of claim 20, wherein the self-antigen is selected from the group consisting of gp100, MART1, MUC1, HER-2, CEA, PSA, prostate membrane specific antigen (PSMA), tyrosinase, tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2), NY-ESO-1, and GA733.

22. The method of claim 16, further comprising administering more than one heterologous or altered antigen that induces an immune response to the native or self-antigen.

23. The method of claim 16, wherein the antigen-presenting cell is genetically modified.

24. The method of claim 23, wherein the genetically modified cell expresses a heterologous or altered antigen corresponding to the native self-antigen.

25. The method of claim 16, further comprising genetically modifying the APC to express a cytokine.

26. The method of claim 16, further comprising genetically modifying the APC to express a co-stimulatory molecule.

27. A method of adoptive immunotherapy, comprising administering to a subject an effective amount of a population of educated, antigen-specific immune effector cells of claim 1.

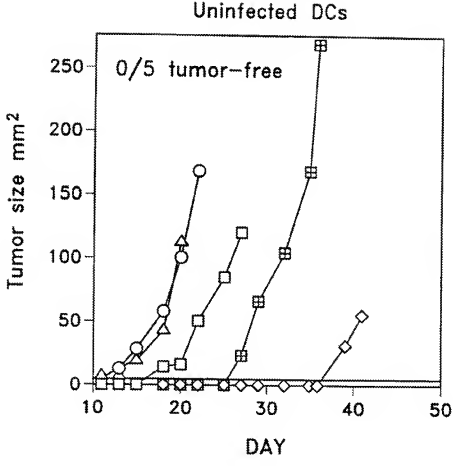
Uninfected DCs
0/5 tumor-free
Tumor size mm2
250
200
150
100
50
0
10
20
30
40
50
DAY

FIG. 1A

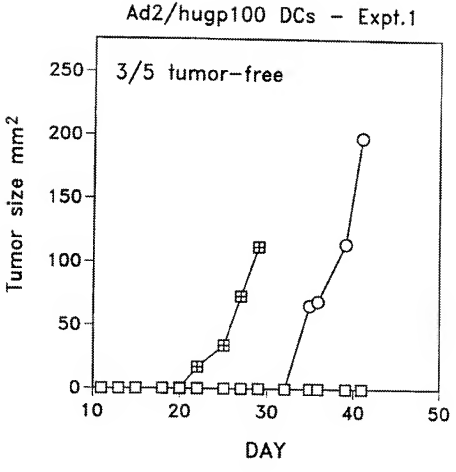
Ad2/hugp100 DCs – Expt.1
3/5 tumor-free
Tumor size mm2
250
200
150
100
50
0
10
20
30
40
50
DAY

FIG. 1B

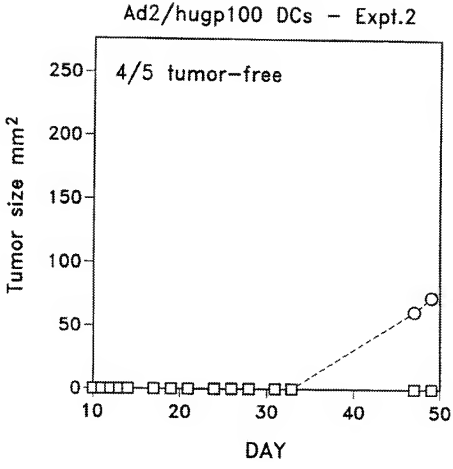
Ad2/hugp100 DCs – Expt.2
4/5 tumor-free
Tumor size mm2
250
200
150
100
50
0
10
20
30
40
50
DAY

FIG. 1C

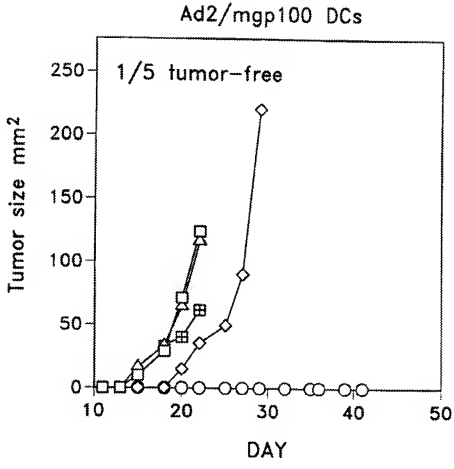
Ad2/mgp100 DCs
1/5 tumor-free
Tumor size mm2
250
200
150
100
50
0
10
20
30
40
50
DAY

FIG. 1D

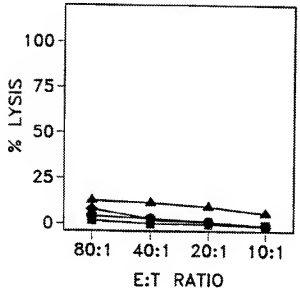

FIG. 2A

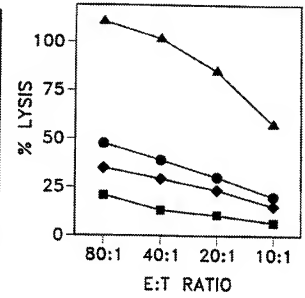

FIG. 2B

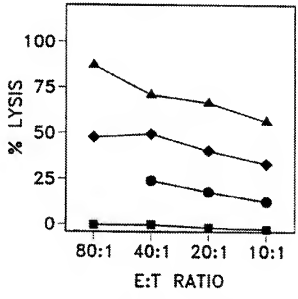

FIG. 2C

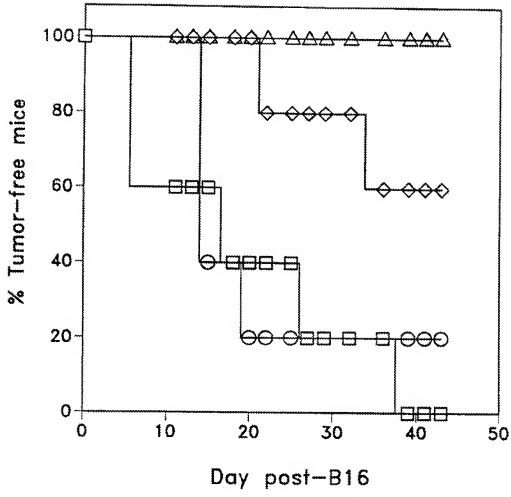

FIG. 3

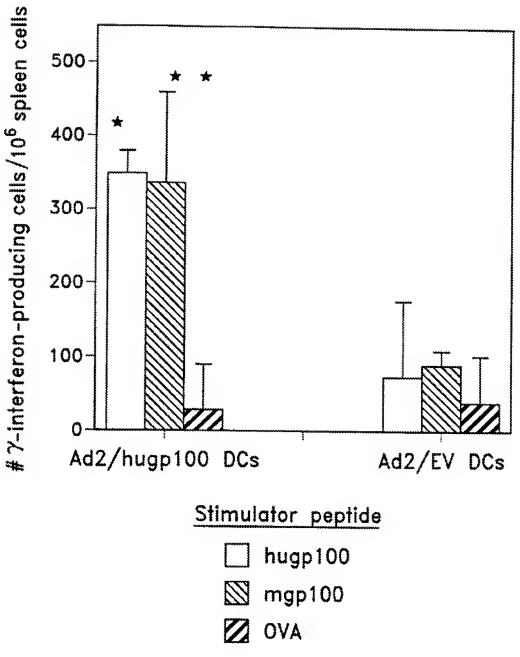
γ-interferon-producing cells/10^6 spleen cells
500
400
300
200
100
0
Ad2/hugp100 DCs
Ad2/EV DCs
Stimulator peptide
hugp100
mgp100
OVA

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06039

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 63/00; A61K 38/02, 39/00; C12N 5/06, 5/10
US CL : 424/184.1, 277.1, 93.7, 435/325, 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 277.1, 93.7, 435/325, 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG medicine index, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOILOLA, L. et al. T-helper epitopes on human nicotinic acetylcholine receptor in myasthenia gravis. Ann. NY Acad. Sci. 1993, Vol. 681, pages 198-218, see entire document, especially pages 199 and 214.	1-4 6-8, 10
X	PACHNER, A.R. et al. An immunodominant site of acetylcholine receptor in experimental myasthenia mapped with T lymphocyte clones and synthetic peptides. Immunol. Lett. February 1989, Vol. 20, No. 3, pages 199-204, see entire document.	1-8, 10, 27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 09 JUNE 1999	Date of mailing of the international search report 13 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer F. PIERRE VANDERVEGT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06039

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BELLONE, M. et al. Experimental myasthenia gravis in congenic mice. Sequence mapping and H-2 restriction of T helper epitopes on the alpha subunits of Torpedo californica and murine acetylcholine receptors. Eur. J. Immunol., 1991, Vol. 21, pages 2303-2310, see entire document..	1-4, 6-8 10, 12
X	INFANTE, A. J. et al. Determinant selection in murine experimental autoimmune myasthenia gravis. Effect of the bm12 mutation on T cell recognition of acetylcholine receptor epitopes. J. Immunol. 01 May 1991, Vol. 146, No. 9, pages 2977-2982, see entire document.	1-8, 10
Y	CHAKRABORTY, M. et al. Preclinical evaluation in nonhuman primates of an anti-idiotypic antibody that mimics the carcinoembryonic antigen. J. Immunotherapy. February 1995, Vol. 18, No. 2, pages 95-103, see entire document, especially abstract.	1-4, 6-8, 10-12, 14-15, 19, 21, 24
X - Y	US 5,648,219 A (MacKAY et al) 15 July 1997, see entire document.	16-18, 20, 22, 23, 25, 26 ----- 19, 21, 24
X	XU, Q. et al. An immunogenic self-peptide for T cells in mice with experimental myasthenia. NY Acad. Sci. 1993, Vol. 681, pages 1-4, see entire document.	1-8, 10, 12
X	PACHNER, A.R. et al. Suppressor T-cell lines and hybridomas in murine myasthenia. NY Acad. Sci. 1986, Vol. 505, pages 619-627, especially page 622.	1-8, 10, 12, 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06039

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 9, 13
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim is drawn to "The method of claim 1," but claim 1 is a product, not a method. Claim 13 is incomplete.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest

☐ No protest accompanied the payment of additional search fees